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Ribosome-associated ncRNAs: An emerging class of translation regulators

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Accumulating recent evidence identified the ribosome as binding target for numerous small and long non-protein-coding RNAs (ncRNAs) in various organisms of all 3 domains of life. Therefore it appears that ribosome-associated ncRNAs (rancRNAs) are a prevalent, yet poorly understood class of cellular transcripts. Since rancRNAs are associated with the arguable most central enzyme of the cell it seems plausible to propose a role in translation control. Indeed first experimental evidence on small rancRNAs has been presented, linking ribosome association with fine-tuning the rate of protein biosynthesis in a stress-dependent manner.

Keywords: non-coding RNA, translation control, lncRNA, ribosome

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Translation Regulation by Non-coding RNAs

Gene expression is a complex and multistep cellular process, where transcription, mRNA export, mRNA degradation, translation, and protein turnover rates represent the major regulatory hubs.[1] Studies measuring the transcriptomes and proteomes of mammalian cells in parallel, demonstrated that for the vast majority of protein coding genes, the transcript levels do not reflect the actual protein levels. Although the correlation is higher as initially reported,[2] this new data highlight that mRNA levels do not represent protein levels and most of the differences could be explained by translation regulation control mechanisms.[3] The same is also true for prokaryal organisms, where no correlation between mRNA and protein copy numbers could be found using a single cell approach in *Escherichia coli*.[4] Regarding the observation that the transcriptome does not entirely correlate with the proteome[1,5] the term 'ribonome' was proposed.[3] The 'ribonome' is defined by the total cellular RNA content and its regulatory factors, including ribosomes and their regulatory non-coding RNAs (ncRNAs).

Translation control utilizing structural features and regulatory sequences within the untranslated regions (UTRs) of messenger RNAs (mRNAs) on the one hand and protein-based targeting of translation initiation on the other hand, are reasonably well understood mechanisms.[6] Since Ambros and coworkers discovered the first micro RNA (miRNA) in 1993, ncRNAs came into focus of translational control.[7,8] Shortly thereafter miRNAs turned out to be a widespread family of endogenous ncRNAs, processed from larger hairpin structured precursors to a length of ~22 nucleotides (nt). mRNAs are the target of miRNAs loaded on the RISC complex, to which they bind by imperfect base-pairing, leading to mRNA decay, translational repression, or sequestration of mRNAs to specific cellular compartments.[9,10] In addition to miRNAs, genome-encoded small interfering RNAs (endo-siRNAs) have been described in a variety of multicellular organisms.[11] While these siRNAs are biochemically indistinguishable from miRNAs, they differ by their origin and mode of action.[12] siRNAs are usually *cis*-encoded and processed from long double-stranded RNAs, also loaded to the RISC complex to be functional.[13] Generally, the 21–23 nt long siRNAs bind to mRNAs by perfect

base-pairing and thereby trigger endonucleolytic mRNA cleavage and degradation.[14] Analogous to miRNAs and siRNAs in eukaryotes, bacterial antisense RNAs have been shown to be the main ncRNA regulators of translation. In general antisense RNAs can be clustered into 2 families, the *cis*- and *trans*- acting small regulatory RNAs (asRNAs).[15] In case of *trans*- encoded antisense RNAs, multiple mRNAs are targeted via imperfect base pairing. In contrast *cis*- encoded antisense RNAs, derived from the opposite strand of the same genomic region, accomplish translation repression of their target mRNAs by perfect complementarity.[15]

All of the above mentioned ncRNA translation regulators (miRNAs, siRNAs, asRNAs) share one common feature: they all target mRNAs. This restricts regulation of protein synthesis typically to specific target messages and thus allows fine-tuning of gene expression in time and space of a defined subset of mRNA transcripts. However, is it also possible to regulate the ribosome, the central enzyme of the translation machinery, directly with ncRNAs? By targeting the ribosome, RNA molecules would allow a fast and direct regulation of protein production. Such a rapid response is important under sudden environmental changes and allows the required massive reprogramming of the gene expression pattern.[16] However, conventional signaling pathways, including the synthesis, degradation or modification of protein factors are comparably time and energy consuming.

A Hitchhiker's Guide to The Ribosome

With the notable exceptions of the bacterial transfer-messenger RNA (tmRNA) and the universally conserved signal recognition particle (SRP) RNA, all functionally characterized ncRNAs capable of regulating protein biosynthesis target the mRNA rather than the ribosome directly. This is unexpected given the central role the ribosome plays during cell metabolism and the assumption that the ribosome evolved in the 'RNA world', where it likely learned receiving regulatory input from non-proteinous co-factors. Thus it is conceivable that such ribosome-bound ncRNAs have survived the evolutionary transition from the 'RNA world' to contemporary biology but have so far escaped the detection in transcriptome screens. The kinetic and energetic advantage of ribosome-bound ncRNA translation regulators compared to protein sensors would be the immediate availability and biological functionality of the ncRNA upon changing environmental conditions without the need of prior production of a costly regulatory polypeptide. While initially ribosome-bound ncRNAs and ncRNA fragments were serendipitously found in mRNA-based RNA-seq approaches as 'contaminants',[17-19] recently more focused studies on the ribosomal ncRNA interactome have been conducted (refs. 20–24 and our unpublished data). A plethora of small and long ncRNAs has been identified to be enriched in the polysomal and sub-polysomal fractions, thus emphasizing their putative roles in translation control. First experimental data support the view that these ncRNA entities do not represent passive hitchhikers of the translation machinery but appear what can be called an emerging class of non-coding ribo-regulators of protein biosynthesis.[22,24,25]

Ribosome-bound small ncRNAs. In our lab we performed targeted transcriptome screens for ribosome-associated ncRNAs (rancRNAs) that potentially regulate protein biosynthesis. Therefore we have applied numerous environmental stress conditions to various model systems spanning all 3 domains of life followed by ribosome preparation, small RNA isolation, and finally RNA-seq analyses. By this approach we have picked up thousands of different small RNA molecules in the size range between 20 and ~300 nt (refs. 21,22, and our unpublished data). The RNAs either originate from intergenic regions of the genomes, and thus represent so far unrecognized ncRNA genes, or they are processed out of functional precursor transcripts such as mRNAs, tRNAs, snoRNAs, SRP RNA, and rRNAs. Post-transcriptional RNA cleavage events have been demonstrated to further expand the spectrum and functionality of transcriptomes.[26,27] In-depth analyses on the fate of these processing products are largely lacking, or are restricted to investigations on RNAi-related *trans*-silencing activities.[26] The rancRNAs in our screens were not only processed from specific sites of the parental RNA, but also showed stress-specific expression or ribosome-association.[21,22,24] Some of these ncRNAs are able to inhibit protein production on the global scale,[22,24] others obviously have a stimulating effect on translation (our unpublished data) (**Fig. 1**). Two ribosome-bound ncRNAs that were investigated in more detail originate from the TRM10 open reading frame in *S. cerevisiae*,[24] or from the 5' parts of valine and alanine tRNAs of the halophilic archaeon *Haloferax volcanii* (ref. 22 and unpublished data). These ncRNAs down regulate protein synthesis on a global level by interacting with the large or small ribosomal subunit, respectively. It is important to note that the mode of action of these 2 examples is different. Whereas the tRNA-derived fragment of *H. volcanii* competes with mRNA binding to the small ribosomal subunit, the ncRNA originating from the *S. cerevisiae* TRM10 mRNA interferes with P-tRNA occupancy (our unpublished data). It was shown that these regulatory events are stress-dependent and occur quickly in response to sudden environmental changes. This highlights the power of ribosome-bound ncRNAs for rapid global translation attenuation. In *S. cerevisiae* we could demonstrate that a ribosome-bound ncRNA is needed for rapid shutdown of global translation and efficient growth resumption under hyperosmotic conditions.[24] Obviously this fast and global attenuation of metabolic activity as a consequence of high salt stress is crucial to open a time window for stress-specific adaptation programs to be established. Both the mRNA-derived fragment in yeast, as well as the tRNA-derived fragments in *H. volcanii* seem to inhibit the translation initiation process. However, there is no reason to assume that translation initiation is the sole step that can be regulated by small rancRNAs. Indeed, certain ncRNA candidates appear to specifically interfere with the elongation phase of protein biosynthesis (our unpublished data) but in principal it is conceivable that every sub-step of the translation cycle

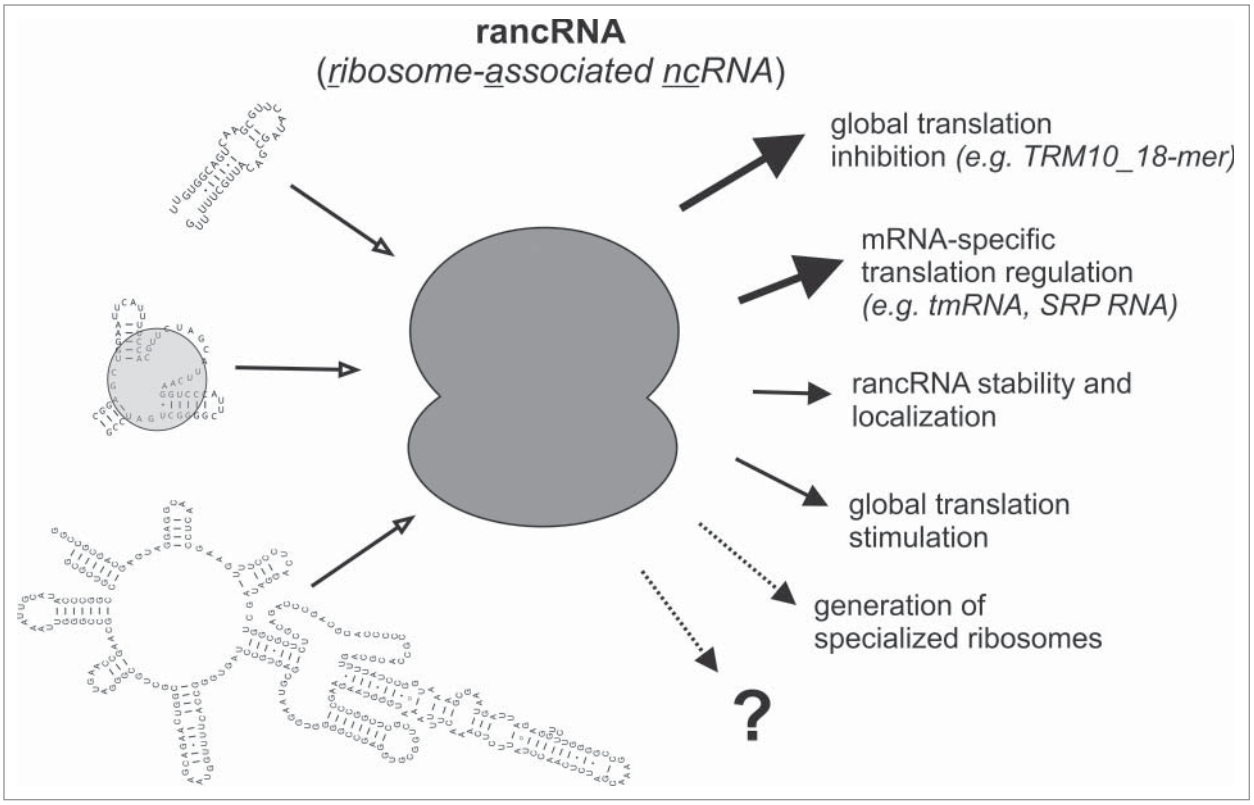

Figure 1. Functional consequences of ncRNA-ribosome interactions. Short or long rancRNAs can target ribosomes (dark gray) either as naked molecules or as RNPs (light gray). As a consequence global (e.g., the yeast TRM10 mRNA-derived 18-mer ncRNA)[24] or mRNA-specific translation regulation can occur. Loading ribosomes on ncRNAs can also affect the cellular stability and/or localization of rancRNAs (ref. 19 and references therein). Size and line thickness of the arrows on the right correspond to experimentally supported (thick and solid), predicted (thin and solid), or in principle possible (dotted) rancRNA functions.

could be affected by ncRNA-mediated regulation.

Besides regulating protein synthesis on a global level, rancRNAs have also the potential to target the translation of specific mRNAs (**Fig. 1**). Two prominent examples for the latter scenario are the bacterial tmRNA and the SRP RNA. tmRNA mediates a unique global quality control system that combines translational surveillance with the rescue of stalled ribosomes.[28] tmRNA specifically recognizes and binds to ribosomes that got stuck on open reading frames (ORFs) due to the absence of stop codons or due to other unproductive pausing events. This ribosome-targeted ncRNA functions as both, mRNA and tRNA, and thus enables ribosome recycling and simultaneously tagging of the incompletely translated protein for degradation.[29] The second example for a well-known mRNA-specific rancRNA is the SRP RNA, which is an integral part of the abundant, cytosolic, and universally conserved SRP ribonucleoprotein (RNP) complex. The SRP is involved in targeting of certain nascent polypeptides to protein-conducting membrane channels, enabling transportation of nascent polypeptide chains across membranes as well as their integration into the membrane itself.[30] Thereby the ncRNA component of the complex (7SL RNA in eukaryotes and 4.5S RNA in bacteria) is not only necessary for binding to the ribosome and recognition of the emerging peptide signal sequence,[31,32] but also for the whole complex assembly and thus represents the functional core of the SRP.[33] Recently an additional function for a 7SL-derived ncRNA has been proposed. The *Alu* RNA (a repetitive element originating from the 7SL RNA) has been suggested to deliver the protein dimer SRP9/14 to the small ribosomal subunit.[34] As a consequence reduced polysome levels were observed resulting in global translation inhibition.[35]

The small rancRNAs are reminiscent of known low molecular weight effectors, such as antibiotics and other secondary metabolites, which have been shown to be capable of tuning the ribosome.[36] The 2 functionally studied small rancRNAs in yeast and *H. volcanii* have been demonstrated to target functional hotspots of the ribosomes and possess K_d values in the low micromolar range, comparable to ribosome-targeted antibiotics. These examples of ribosome-bound small ncRNAs likely represent only the forefront of a so far largely elusive class of translation regulators and can pave the way for novel mechanisms to be uncovered.

Ribosome-bound long ncRNAs. Long ncRNAs (lncRNAs) have recently received considerable attention in the field. This class of ncRNA molecules is vaguely defined by the length range of >200 nt to several kilobases.[37] Initially lncRNAs have been connected to chromosome dosage compensation in mammals (*Xist* RNA)

and regulation of imprinting (e.g. *HOTAIR* RNA). Recent years have witnessed a burst of lncRNA identification (primarily by bioinformatic means) and have expanded the scope of lncRNA functions to transcription enhancer, miRNA sponging, RNA turnover, or translation control roles (reviewed in refs. 37, 38). These capabilities of lncRNAs in turn affect the regulation of crucial cellular processes such as embryogenesis, cell cycle, maintenance of pluripotency, apoptosis, and differentiation. In general lncRNAs share common features with mRNAs, such as transcription by polymerase II, splicing, 5'-capping, and 3'-polyadenylation. What distinguishes them from genuine mRNAs is the lack of reasonably long and evolutionarily constrained ORFs, the predominant nuclear localization, and the lack of encoded peptide fragments detectable in mass spectrometry studies. Recent ribosome profiling, translating ribosome affinity purification (TRAP), as well as polysome profiling approaches, however, presented evidence that some lncRNAs are in fact cytoplasmic and associate with ribosomal and poly-ribosomal fractions.[17,20,23,39-41] This raises the possibility that lncRNAs are ribosome-bound to fine-tune the speed or specificity of the translation machinery (**Fig. 1**). It has been suggested that lncRNAs decorated with multiple ribosomes would be a means for titrating out and storing ribosomes for later use.[23] Alternatively it has been proposed that lncRNAs pair sequence specifically with certain mRNAs promoting[42] or inhibiting[43] their translation. Strictly speaking, the latter anti-sense lncRNAs are in fact not genuine rancRNAs since they associate with polysomes via their hybridization with mRNAs. On the other hand polysomal lncRNAs might in fact co-sediment with translating ribosomes because they actually encode proteins or short peptides. The currently available data fueled an intense discussion about the possible protein coding potential of several lncRNAs[19,44] and called into question the annotation of non-protein-coding transcripts.[37,38] A study in zebrafish has shown that up to 45% of previously proposed lncRNAs possess detectable ORFs and therefore might actually represent genuine mRNAs,[19] thus stressing the fact that the protein/peptide coding potential of lncRNAs has been underestimated. In support of this view, 2 very recent whole human proteome studies identified up to ~450 peptides to be encoded in annotated lncRNAs, pseudogenes, and other transcripts of uncertain coding potential.[45,46] It therefore appears that conventional gene annotations over-estimated the number of lncRNAs in vertebrate genomes. Nevertheless the ribosome association of genuine lncRNAs implies an attractive possibility for translation control and awaits further investigations.

Conclusion & Outlook

Thousands of putative ribosome-associated ncRNAs (rancRNAs) have been recently identified, yet not all of them are expected to alter the performance of the ribosome. It is possible that some, or even the majority, of these RNA molecules are ribosome-bound by unspecific interactions and thus represent biological noise. Others might be ribosome-bound because the ribosome is in a state that can be referred to as 'default translation initiation' mode (**Fig. 1**). This might represent the basal program of the translation machinery attempting to spuriously bind initiation codons on all encountered cytoplasmatic RNAs.[23] On the other hand, first experimental evidence on some small rancRNA molecules has been presented that suggest indeed translation control functions.[22,24,25] These first examples demonstrated a very rapid global attenuation of protein production in a stress-dependent manner (**Fig. 1**). Furthermore, mRNA-specific effects on protein biosynthesis by rancRNAs have also been observed with the well characterized SRP RNA and tmRNA as role models for this subclass of ribo-regulators. Future work will need to clarify whether or not ribosome-bound ncRNAs are capable of eliciting additional regulatory cues to the translation machinery and thus further expand the known repertoire of translation regulation and ncRNA biology (**Fig. 1**).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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